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Escherichia coli Membranes Depleted of SecYEG Elicit SecA-Dependent Ion-Channel Activity but Lose Signal Peptide Specificity

Bor-Ruei Lin · Ying-Hsin Hsieh · Chun Jiang · Phang C. Tai

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Abstract We have developed a sensitive method to detect the opening of SecA-dependent, protein-conducting channels in *Xenopus* oocytes. In this study, we determined the ionic current activities of the SecA-dependent channel from membrane vesicles depleted of SecYEG. We found that these SecYEG-depleted membranes produced SecA-dependent ionic currents in the oocytes, as did membranes containing SecYEG. However, reconstituted membranes depleted of SecYEG required higher concentrations of SecA to elicit ionic currents like those in membranes containing SecYEG. In contrast to membranes containing SecYEG, the proofreading capacity of signal peptides was lost for those membranes lacking SecYEG. These findings are consistent with loss of signal peptide specificity in channel activity from membranes of SecY suppressor or SecY plug domain mutants. The signal peptide specificity of the reconstituted membranes, like SecA-liposomes, can be restored by the addition of SecYEG proteoliposomes. On the other hand, the channel activity efficiency of reconstituted membranes was fully restored, while SecA-liposomes could only be partially enhanced by the addition of SecYEG, indicating that, in addition to SecYEG, other membrane proteins contribute to the efficiency of channel activity. The SecA-dependent channels in membranes that lacked SecYEG also lost ion selectivity to monovalent cations but retained selective permeability to large anions. Thus, the electrophysiological evidence presented here indicates that SecYEG is not obligatory for the channel activity of *Escherichia coli* membranes, as previously shown for protein translocation, and that SecYEG is important for maintenance of the efficiency and specificity of SecA-dependent channels.

Keywords Electrophysiology · Ion channel · Mechanism of transport protein · Biochemistry/Molec. Biology

B.-R. Lin and Y.-H. Hsieh contributed equally to this work.

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B.-R. Lin · Y.-H. Hsieh · C. Jiang · P. C. Tai (✉)
Department of Biology, Petit Science Center, Georgia State University, 161 Jesse Hill Dr., Suite 490, Atlanta, GA 30303, USA
e-mail: biopct@gsu.edu

Y.-H. Hsieh
e-mail: yhsieh3@student.gsu.edu

Introduction

The characteristics of the SecA and SecYEG complex involved in *Escherichia coli* protein translocation have been studied extensively over the last few decades. SecA has been widely viewed as a peripheral ATPase protein that is able to cycle on and off the membranes during protein translocation. By hydrolyzing ATP as the energy source, it has been shown that SecA inserts part of its domain into the SecYEG protein-conducting channel and thereby drives precursors across the translocase complex (Manting and Driessen 2000; Mori and Ito 2001; Veenendaal et al. 2004). However, the centrality of the SecYEG complex as the only protein-conducting channel has also been brought into question. It has been found that certain precursor proteins can be translocated in vitro with membranes depleted of either SecY or SecE (Watanabe et al. 1990; Yang et al. 1997a, b) or reconstituted membranes with the removal of SecYEG (Hsieh et al. 2011). Moreover, the SecYEG complex may not form a pore large enough for protein translocation (Van den Berg et al. 2004).

The roles of SecA in protein-conducting channels have been evaluated by different research groups. Studies have shown that SecA is permanently embedded in *E. coli* cytoplasmic membranes (Chen et al. 1996) and capable of forming ring-like pore structures in the presence of anionic phospholipids (Wang et al. 2003). Through the in vivo use of sulfhydryl domain-specific labeling, the membrane-embedded SecA has been shown to possess multiple domains that are oriented toward, and exposed to, the periplasmic side of the membrane (Jilaveanu and Oliver 2007). It has also been demonstrated that the concentration of SecA is increased in cells to compensate for several defects in protein translocation (Cabelli et al. 1988; Fandl et al. 1988; Fandl and Tai 1987; Kusters et al. 1992). More recent in vitro studies have shown that SecA may function as a dimer for protein translocation (Jilaveanu and Oliver 2006; Jilaveanu et al. 2005; Wang et al. 2008). Recently, we reported that SecA liposomes alone without SecYEG participate in ion-channel activity (and protein translocation), indicating that SecA is essential and sufficient for channel activity (Hsieh et al. 2011). However, this channel activity is less efficient than that of *E. coli* membranes with SecYEG and requires more SecA and additional ATP, suggesting that other membrane components may contribute to the SecA/liposomal protein-conducting channel.

We have recently developed a sensitive method to measure the activity of protein-conducting channels in *E. coli* membranes by injecting inverted bacterial membrane vesicles into *Xenopus* oocytes (Lin et al. 2006). In so doing, we have found that ionic currents through such membranes were strictly SecA-dependent and could be blocked by SecA inhibitors such as sodium azide and nonhydrolyzable ATP analogs. Using this electrophysiological method, we were able to examine the central components of protein-conducting channels in bacterial membranes and determine the function of Sec proteins at various steps in protein translocation.

In this study, we provide new evidence for the SecA-mediated, protein-conducting channel in the absence of the SecYEG complex using similar electrophysiological measurements. Ionic currents were recorded from oocytes injected with *E. coli* membrane vesicles in which SecYEG were depleted or removed. The currents were inhibited by puromycin, which removed nascent peptides in the oocytes, and subsequently restored by addition of either wild-type LamB signal peptides or proOmpA precursor proteins. Defective LamB signal peptides or unfolded mature OmpA protein were also capable of producing the ionic currents in membranes lacking SecYEG. The observations are consistent with our previous findings in membranes containing SecY mutants (Hsieh et al. 2011), suggesting a loss of proofreading function for signal sequences in the absence of SecY. Moreover, such proofreading capability can be fully restored by supplementing purified SecYEG. Taken together, the electrophysiological studies presented here, when combined with previous biochemical and physical evidence (Chen et al. 1996, 2007; Wang et al. 2003; Yang et al. 1997a, b), indicate that SecYEG is not obligatory for SecA-dependent ionic currents. The data further suggest an important role of SecYEG in the maintenance of protein channel efficiency and specificity.

Materials and Methods

Bacterial Strains *E. coli*

K12 strain MC4100 was from J. Beckwith (Silhavy and Beckwith 1983), and BA13, a derivative of MC4100, was from D. Oliver (Oliver and Beckwith 1982a). MC1000 was from J. Beckwith (Chen et al. 1996). BL21(DE3)/pT7-SecA, for overproducing the SecA protein by the T7 promoter expression system, was from D. Oliver (Cabelli et al. 1988). *E. coli* strains D10-1 and D10-3 are lab stocks as described (Fandl et al. 1988). PrlA665 was a gift from T. Silhavy (Emr et al. 1981). All cells were grown in a buffered Luria–Bertani medium LinA with 0.5 % glucose with aeration (Tai et al. 1991).

Reagents and Chemicals

All chemicals are of reagent grade and were obtained from Sigma-Aldrich (St. Louis, MO) or other commercial sources.

Purification of SecA, proOmpA and LamB Signal Peptides

SecA was purified from BL21 (DE3)/pT7-SecA as described (Cabelli et al. 1988). Purified proOmpA and OmpA were prepared as described (Chen et al. 1987, 1996). Protein amounts were determined using a Bio-Rad (Richmond, CA, USA) assay kit with bovine serum albumin as standard. Wild-type LamB signal peptide (MMITLRKLPLA VAVAAGVMSAQAMA) and LamB deletion mutant signal peptide (MMITLRKLP—VAAGVMSAQAMA) were gifts from Lila Gierasch (Lin et al. 2006).

Preparation of SecYEG-Depleted Membranes

Membranes from wild-type *E. coli* MC1000, MC4100 (Oliver and Beckwith 1982b) and D10-3 lacking F_1F_o-ATPase and OmpT were prepared as described (Tai et al. 1991). SecA-depleted membrane vesicles from the *secA* amber mutant strain BA13 were prepared as described (Tai

et al. 1991). SecYEG$^-$ membrane vesicles were prepared from strain PS289 according to the procedures described by Yang et al. (1997b). The residual amount of SecYEG used in this study is <1 %.

Removal of SecYEG from Reconstituted Membranes

Reconstituted membranes were prepared as described previously, which resulted in the removal of >99 % of the SecY (Nicchitta and Blobel 1990; Watanabe et al. 1990) with modifications. Cytoplasmic membranes were obtained from either the *E. coli* strain D10-3 or BA13 by two layers of sucrose gradients (Tai et al. 1991), washed by high-salt buffer (0.4 M sucrose, 1 M potassium acetate, 20 mM triethanolamine hydrochloride [pH 7.5], 1.5 M magnesium acetate and 1 mM EDTA) and resuspended at a final concentration of 3–4 mg/ml. Sodium cholate was added at a final concentration of 1 %. The mixture was incubated on ice for 1 h and then centrifuged in a Beckman (Fullerton, CA, USA) TLA-100.3 rotor at 90,000 rpm for 30 min. The supernatant was collected and treated with 0.75 % sodium cholate in at least double volume of high-salt buffer and then placed on ice for 1 h, followed by centrifugation in the TLA-100.3 rotor at 90,000 rpm for 30 min. The supernatant was collected and dialyzed with a Spectra-Por 1 dialysis membrane (Spectrum Medical Industries, Houston, TX) at room temperature against 500–1,000 volumes of dialysis buffer (0.25 M sucrose, 0.4 M potassium acetate, 20 mM triethanolamine hydrochloride [pH 7.5], 1.5 mM magnesium acetate and 1 mM EDTA) overnight. The dialysis membrane was first boiled for 10 min in 1 % $NaHCO_3$ and 1 mM EDTA, then boiled in deionized H_2O for 10 min and cooled on ice before use. After dialysis, reconstituted membrane vesicles were collected by centrifugation in the TLA-100.3 rotor at 90,000 rpm for 15 min. Pellets were suspended with stirring in DTK buffer (1 mM dithiothreitol, 10 mM Tris–HCl [pH 7.6], 50 mM KCl), and the concentration was determined by measuring the OD_{280}/OD_{260} ratio with the Bio-Rad SmartSpec 3000. Such reconstituted membranes contained <1 % SecYEG.

Xenopus Oocyte Preparation and Injection

Oocytes were obtained from *Xenopus laevis* (Mao et al. 2004; Xu et al. 2001). Frog surgery and sample injection were performed as described previously (Lin et al. 2006). Samples containing *E. coli* membranes, SecA and precursors were premixed together and then injected into the dark side of oocytes by the Nanojector II microinjection system (Drummond Scientific, Broomall, PA, USA). Injected oocytes were incubated at 23 °C for 2.5–3.0 h, and outward currents were recorded at room temperature with the two-electrode voltage-clamp technique using KCl as the major conducting ion in the bath solution, unless indicated otherwise. The final amounts or concentrations of sample injected were membranes (60 ng), wild-type and defective LamB signal peptides (1 pmol) and puromycin (4 mM) as standardized concentrations unless otherwise indicated. Estimates of injected sample concentrations in oocytes were made based on the average volume of oocytes at 500- and 50-nl injection volumes (Lin et al. 2006).

Voltage-Clamp Measurements

Ionic currents were recorded using the two-electrode voltage-clamp technique, as previously described (Lin et al. 2006). In brief, whole-cell currents were studied on oocytes 3 h after injection. Two-electrode voltage-clamp measurements were performed using an amplifier (Geneclamp 500; Axon Instruments, Foster City, CA) at room temperature (~24 °C). Cells were impaled using electrodes filled with 3 M KCl. One of the electrodes (1.0–2.0 MΩ) served as a voltage recorder and was connected to the HS-2 x1L headstage, while the other electrode (0.3–0.6 MΩ) was used for current recording and connected to the HS-2 x10MG headstage. Oocytes were accepted for further experimentation only if they did not show leakage in membrane currents. Current records were low pass-filtered (Bessel, 4-pole filter, 3 dB at 5 kHz), digitized at 5 kHz (12-bit resolution) and stored on a computer disk for later analysis (pClamp 6.0.3, Axon Instruments). Junction potentials between bath and pipette solutions were appropriately nulled before impaling the cell.

Data Analysis

Data are presented as mean ± SE. Differences in means were tested with Student's *t* test for a pair of data and ANOVA for three groups or more. Differences were accepted as significant at $p \leq 0.05$.

Results

Detection of Ionic Currents from Injection of Membranes Depleted of SecYEG

Ionic currents were studied with and without SecYEG-depleted membranes. In these membranes, SecYEG proteins were below detectable levels and SecA was elevated by sixfold as determined immunologically (Yang et al. 1997b; Yi et al. 2003). Concentration-dependent increases in ionic currents were produced with increased amount of membranes under the condition of SecYEG depletion (Fig. 1a). In comparison with wild-type membranes, these currents saturated at 6.6 ± 0.8 μA ($n = 10$) remained blocked by AMP-PCP, a nonhydrolyzable ATP analog for

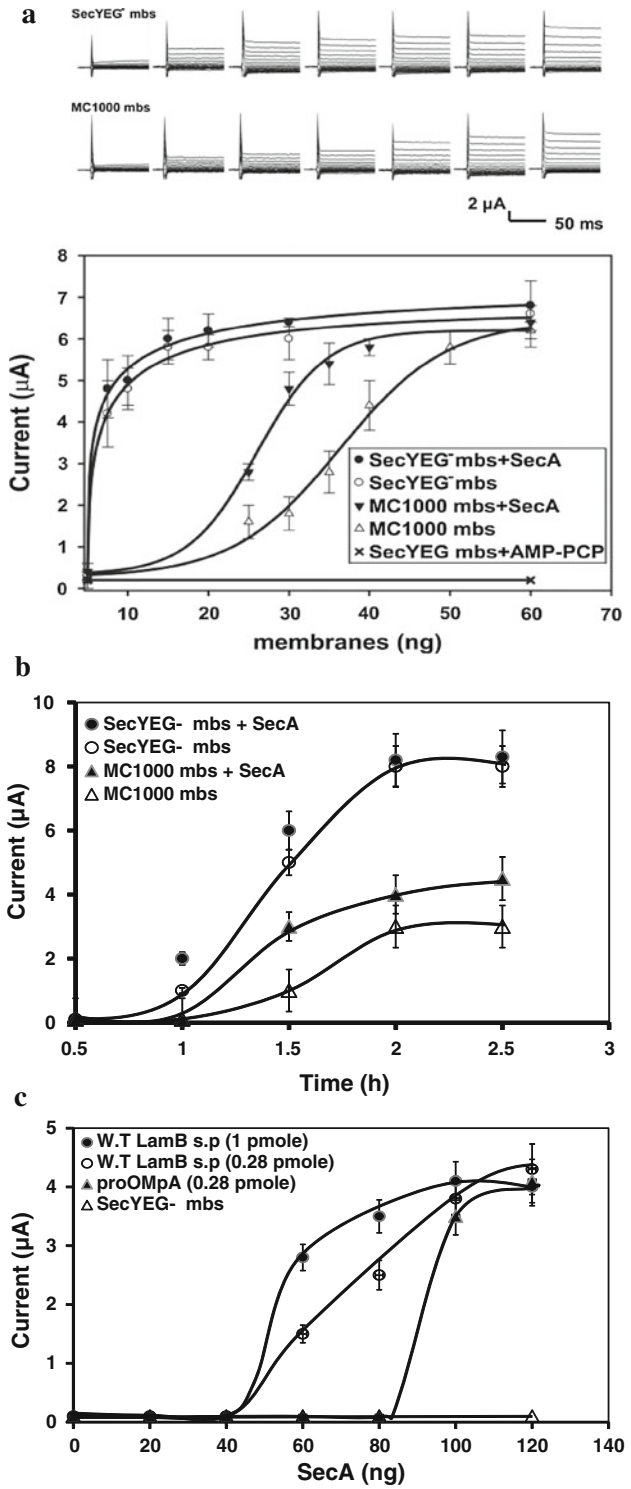

Fig. 1 Channel activities of SecYEG$^-$ and MC1000 membranes. **a** Ionic currents were measured from different amounts of SecYEG$^-$ or MC1000 membranes injected into oocytes, and SecA (5 ng) or AMP-PCP (4 mM) was coinjected with membranes where indicated. Tracings of current recording of different amounts of injected membranes are shown above with the same scale. **b** SecYEG$^-$ or MC1000 membranes (30 ng) were injected with or without SecA in the time-course experiment in the absence of puromycin. **c** Different amounts of SecA were coinjected with SecYEG$^-$ membranes and wild-type LamB signal peptides or pOmpA in the presence of puromycin, which acts to block the oocyte-endogenous signal peptides

inhibition of SecA activity (Fig. 1a), and were enhanced with the additional SecA (Fig. 1b). Indeed, the SecYEG$^-$ membranes showed even higher current activity compared to wild-type MC1000 membranes (Fandl et al. 1988).

We found that oocyte-endogenous signal peptides can open protein-conducting channels in *E. coli* membranes and that such activity can be inhibited by puromycin, which functions to block the synthesis of oocyte-endogenous precursor proteins bearing signal peptides (Lin et al. 2006). Similarly, there were no detectable ionic currents in the presence of puromycin (Fig. 1c). In our previous finding, 20 ng of SecA was sufficient to recover the ionic currents with wild-type LamB signal peptides in wild-type *E. coli* membranes (Lin et al. 2006). However, higher concentrations of exogenous SecA were needed to restore ionic currents in SecYEG$^-$ membranes with either LamB signal peptides or OmpA precursor proteins (Fig. 1c).

SecA Stimulates Ionic Currents on Reconstituted Membranes with SecYEG Removed In Vitro

Although SecYEG$^-$ membranes are good for studying channel activity without SecYEG, it is likely that stress components were induced during their depletion in cells (Ruiz and Silhavy 2005). Thus, we reconstituted SecYEG$^-$ membranes from *E. coli* D10-3, which was an OmpT$^-$ strain, and SecY was removed by sodium cholate precipitation (Watanabe et al. 1990; Zhong et al. 1996). The reconstituted membranes contained a negligible amount (<1 %) of SecYEG, while other Sec proteins, SecD, SecF and YidC, were all retained (Fig. 2a). To determine the role of SecA in the reconstituted membranes, we prepared reconstituted membranes lacking SecA as well as those lacking SecYEG (RE-BA13). We were able to detect ionic currents from these membranes only in the presence of additional exogenous SecA (Fig. 2b). The recording of currents from SecYEG$^-$ reconstituted membranes (RE-BA13) required higher amounts of SecA (at least 60 ng) than those SecYEG$^-$ membranes, suggesting that these ionic currents are strictly SecA-dependent and that the SecA-dependent currents are less efficient in the absence of SecYEG (Hsieh et al. 2011). The sharp rise of channel activity with increasing SecA further suggested a cooperative aspect to the ability of SecA to conduct current at critical concentrations.

Loss of Channel Proofreading and Efficiency in the Absence of SecYEG

SecY is suggested to provide the proofreading function for the signal peptides (Osborne and Silhavy 1993). We

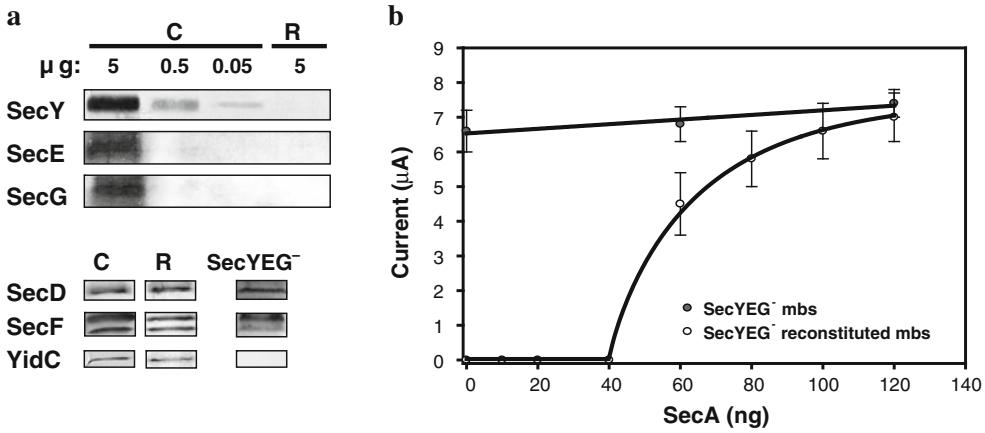

Fig. 2 Reconstituted SecYEG$^-$ membranes are active. **a** SecY, SecE and SecG were examined by Western blot analysis with *E. coli* strain D10-3 crude membrane extracts and reconstituted membranes. Reconstituted membranes contained <1 % of SecY content and nondetectable SecE and SecG. Antibodies against SecD, SecF and YidC were also used for these two membrane preparations. SecYEG$^-$ membranes = 5 μg. *C* crude membranes, *R* reconstituted membranes. **b** Different amounts of SecA were injected with SecYEG$^-$ reconstituted membranes or SecYEG$^-$ membranes in the absence of puromycin

investigated whether removal of SecYEG in the membranes had any effect upon the proofreading function of the ion channel activity in the oocyte system. In our previous study, neither mature OmpA protein nor defective LamB signal peptides were capable of stimulating channel activity from wild-type membranes containing SecYEG (Lin et al. 2006). Here, we find that the ionic currents elicited by proOmpA or wild-type LamB signal peptides were almost as effective in both the SecYEG-depleted membranes (Fig. 3a) and reconstituted BA13 membranes (Fig. 3b) as they were in wild-type MC4100 membranes. Moreover, membranes lacking SecYEG also produced ionic currents that responded to unfolded mature OmpA as well as defective Lam B signal peptide stimulation to remove oocyte endogenous nascent peptides, even in the presence of puromycin (Fig. 3a, b). These results suggest that the proofreading function in these membranes had been lost. Similar results were also observed in SecY suppressor mutant PrlA665 membranes (Supplemental Fig. 1) and the SecY plug mutant (Hsieh et al. 2011). The increased channel activity that was induced by the mature OmpA protein or LamB defective mutant is in accordance with previous in vitro translocation assay results (Emr et al. 1981) and with SecA-liposomal assays (Hsieh et al. 2011).

SecYEG Fully Restored Channel Specificity and Efficiency

Having shown that membranes without SecYEG lost their ability to proofread signal peptides, the next question was whether this proofreading function could be restored in either SecYEG-depleted membranes or reconstituted BA13 membranes by coinjection of purified SecYEG proteoliposomes. In addressing this question, we found that such reconstituted membranes when coinjected with SecYEG proteoliposomes in the presence of puromycin (Fig. 4a, b) failed to produce ionic currents unless SecA was also added, along with the wild-type precursors (pOmpA) or wild-type LamB signal peptide (LamB WT). Moreover, there were no detectable ionic currents when mature OmpA or LamB defective mutant (LamB DM) was added, indicating that these reconstituted membranes had regained their signal peptide specificity following the addition of SecYEG.

We also used reconstituted PrlA665 membranes (RE-PrlA665) or reconstituted SecY plug mutant membranes (RE-SecY plug mutant) in which the SecY variants had been removed to investigate if the proofreading function of these membranes could be restored by exogenous SecYEG proteoliposomes. Both unfolded mature OmpA and defective LamB signal peptides elicited ionic currents from these membranes (Fig. 5a, b), which proved to be as active as proOmpA or wild-type LamB signal peptides. Addition of SecYEG proteoliposomes with SecA to the RE-PrlA665 membranes or RE-SecY plug mutant membranes restored their proofreading ability to discriminate between proOmpA and mature OmpA as well as wild-type and defective LamB signal peptides (Fig. 5a, b), indicating that such membranes were no longer responsive to mature OmpA or defective signal peptide.

Furthermore, the addition of SecYEG proteoliposomes was also shown to increase ionic currents in reconstituted BA13 membranes (Fig. 6). Indeed, the presence of SecA RE-BA13 membranes with added SecYEG proteoliposomes was able to restore channel activity almost to the same level as wild-type BA13 membranes containing intrinsic SecYEG. Similarly, addition of SecYEG proteoliposomes elicited higher ionic currents to RE-BA13 membranes (Fig. 6).

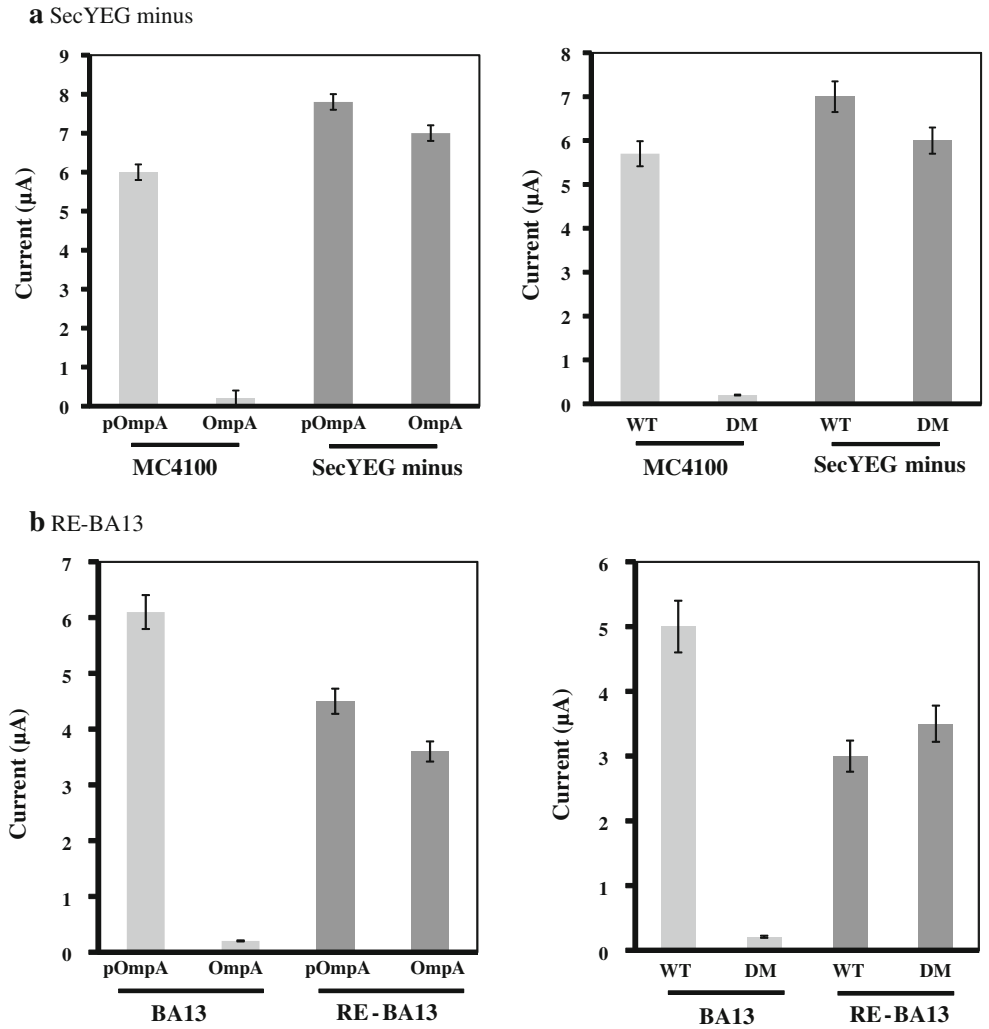

Fig. 3 Loss of proofreading function for SecYEG$^-$ membranes and reconstituted BA13 membranes. **a** SecYEG$^-$ membranes or (**b**) reconstituted BA13 membranes (*RE-BA13*) were injected with precursors (*pOmpA*), mature precursors (*OmpA*), wild-type LamB signal peptides (*WT*) or defective mutant LamB signal peptides (*DM*) in the presence of SecA (60 ng) and puromycin

These results suggest that the proofreading function of membranes lacking SecYEG can be restored by exogenous SecYEG proteoliposomes and that SecYEG is not only responsible for proofreading ability but also important for the efficiency of channel activity. Similar results were found when SecA liposomes were coinjected with SecYEG proteoliposomes, although more SecA was needed for active ionic currents and the restoration of activity was not nearly to the same extent (Fig. 6). These findings indicate that membrane proteins other than SecA/SecYEG also contribute to the higher efficiency of channel activity.

The Protein-Conducting Channel without SecYEG Loses Selectivity for Monovalent Cations

We have previously shown that the protein-conducting channels of wild-type membranes have the capability to discriminate ions according to size (Lin et al. 2006). To examine the effect of SecYEG on ion selectivity, we performed experiments in which the K^+ or Cl^- in the extracellular solution was replaced with Na^+, *N*-methyl-D-glucamine ($NMDG^+$), glutamate$^-$ or gluconate$^-$. The molecular weights of these cations and anions were K^+ (39.1 Da), Na^+ (23.0 Da), $NMDG^+$ (195.2 Da), Cl^- (35.5 Da), glutamate$^-$ (147.1 Da) and gluconate$^-$ (198.8 Da). Membranes depleted of SecYEG$^-$ or reconstituted membranes without SecYEG were injected with SecA and wild-type LamB signal peptides, and the reversal potentials and current amplitude were measured for each conductive ion in the bath solution 2–3 h after injection. According to the direction of ionic movements, inward currents were studied for cations, while outward currents were examined for anions.

Complete replacement of K^+ in the extracellular solution with Na^+ demonstrated only moderate changes in both

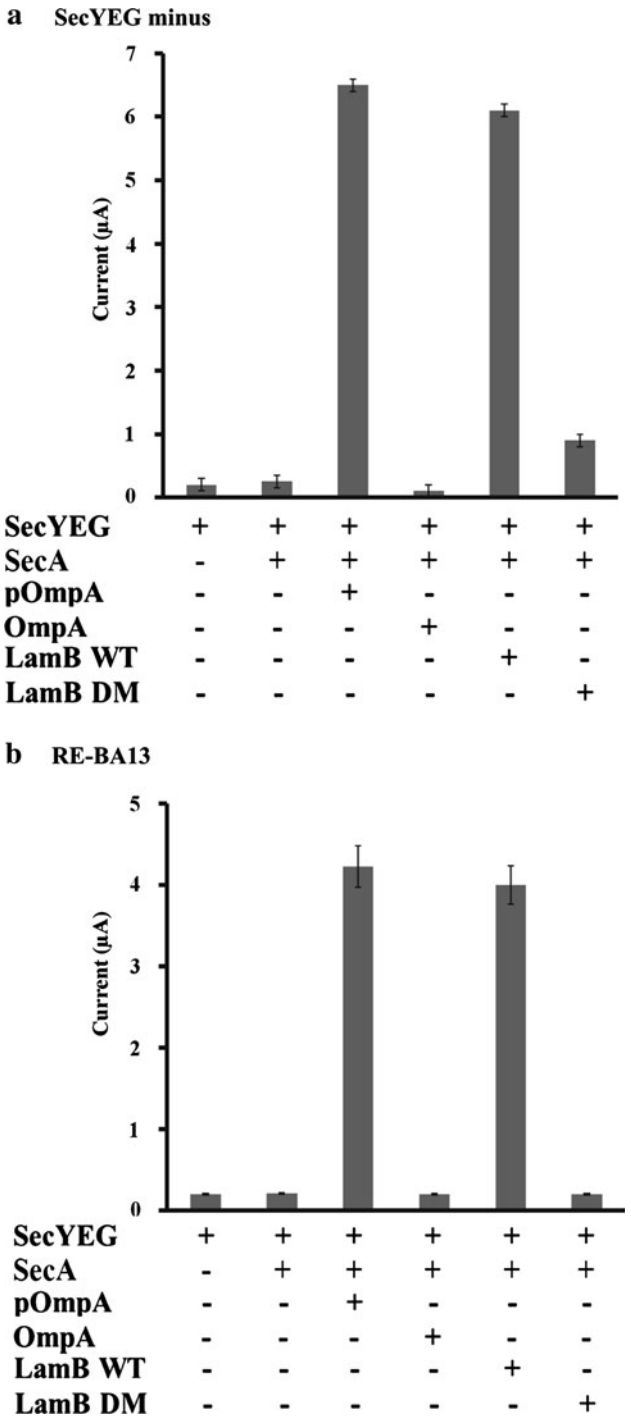

Fig. 4 Restoration of proofreading function of SecYEG$^-$ membranes or reconstituted BA13 membranes by SecYEG proteoliposomes. SecYEG proteoliposomes (30 ng) were coinjected with (**a**) SecYEG$^-$ or (**b**) reconstituted BA13 membranes (*RE-BA13*) and SecA, proOmpA, OmpA, wild-type LamB or defective mutant of LamB signal peptides where indicated in the presence of puromycin

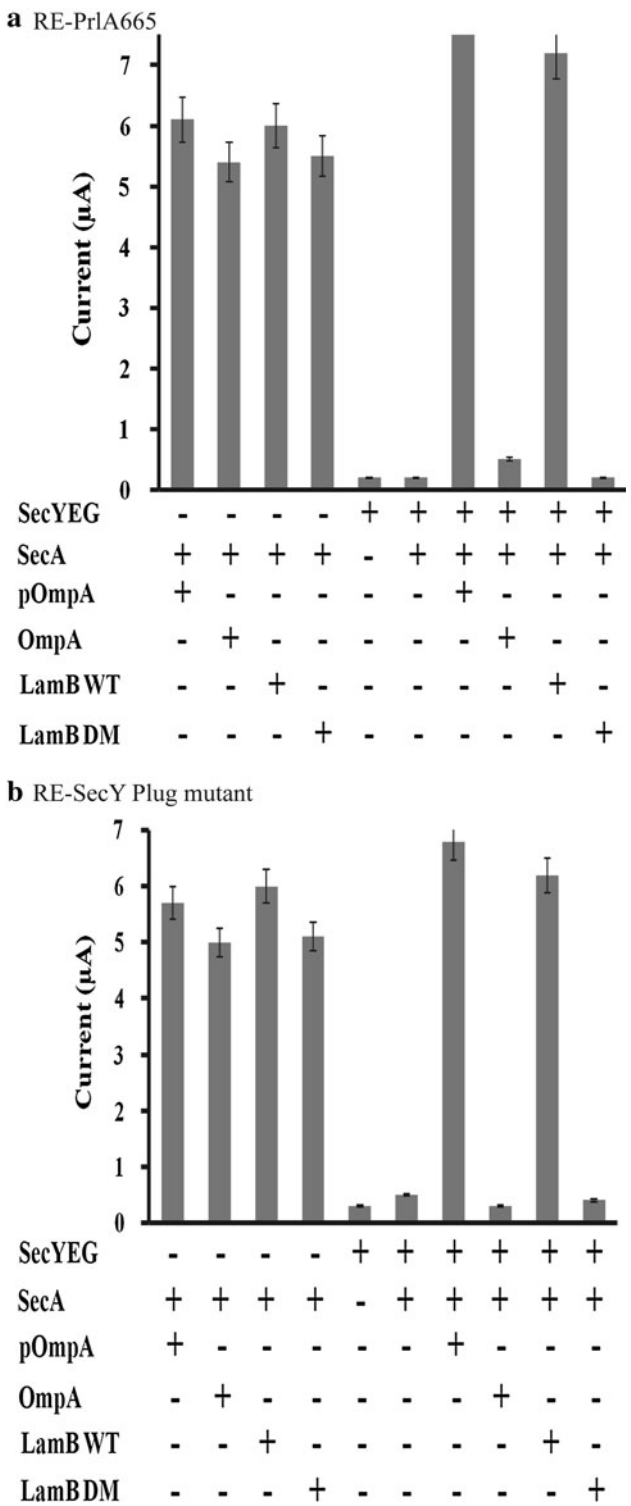

Fig. 5 Restoration of proofreading function in reconstituted membranes of SecY mutants. SecYEG proteoliposomes (30 ng) were coinjected with SecA, precursors or signal peptides with reconstituted membranes of **a** SecY suppressor mutant PrlA665 or **b** SecY plug-deletion mutant in the presence of puromycin

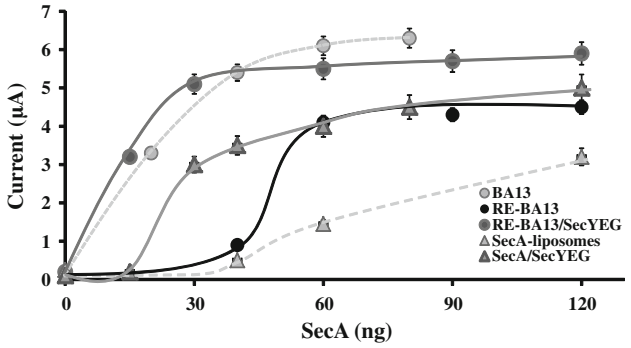

Fig. 6 SecYEG increases channel activity efficiency in reconstituted BA13 membranes or in SecA liposomes. Different amounts of SecA were injected with BA13 membrane (*gray circle*), RE-BA13 (*black circle*) or RE-BA13 membranes plus SecYEG proteoliposomes (*dark gray circles*). SecA liposomes (*light gray triangles*) were prepared by sonication of *E. coli* total lipid extracts. SecA liposomes together with SecYEG proteoliposomes (*dark gray triangles*) were injected with ATP (2 mM) and Mg^{2+} (1 mM) in the presence of SecA and the precursors

reversal potentials and current amplitudes in either the $SecYEG^-$ membranes or the reconstituted $SecYEG^-$ membranes (Table 1; Supplemental Fig. 2a, b), suggesting that all the channels are about equally permeable to K^+ and Na^+. A clear shift in the reversal potential and a reduction in the current amplitude occurred in wild-type membranes when $NMDG^+$ was substituted for K^+ (Table 1). However, no major change in reversal potentials and current amplitude were observed in $SecYEG^-$ membranes or in reconstituted $SecYEG^-$ membranes under similar ion substitution conditions (Table 1; Supplemental Fig. 2a, b). Since the extracellular Cl^- concentration remained the same, these results indicate that, in the absence of SecYEG, the SecA-dependent channels lost selective permeability to the tested cations. Similar ion substitution experiments were performed for other anions. The results showed that the SecA-dependent, protein-conducting channels without SecYEG showed a clear shift in reversal potentials when Cl^- was replaced by $glutamate^-$ or $gluconate^-$ (Table 1).

Based on these reversal potentials, we calculated the relative ion permeability of each of the different cations and anions that were tested (Table 2). The relative permeability of $NMDG^+$ to K^+ is close to 1 for the SecYEG-depleted membranes and reconstituted SecYEG membranes, suggesting that both these membranes become less selective for cations. In contrast, the SecYEG-depleted membranes and reconstituted SecYEG membranes remained selectively permeable to Cl^- over $glutamate^-$ or $gluconate^-$ (Table 2).

Discussion

We have shown that ionic currents can be observed through membranes in the absence of detectable SecYEG

Table 1 Reversal potentials and ionic currents measured from different cations or anions

	MC4100 mbs[a]		$SecYEG^-$ mbs		Reconstituted $SecYEG^-$ mbs	
	Reversal potential (mV)	Current (μA)	Reversal potential (mV)	Current (μA)	Reversal potential (mV)	Current (μA)
Cations						
K^+	−20.2 ± 2.0 (n = 8)	1.5 ± 0 1 (n = 8)	−21.3 ± 3.1 (n = 20)	1.9 ± 0.1 (n = 13)	−32.4 ± 0.1 (n = 15)	2.2 ± 0.1 (n = 10)
Na^+	−21.2 ± 2.1 (n = 8)	1.1 ± 0.3 (n = 8)	−22.3 ± 2.6 (n = 11)	2.0 ± 0.1 (n = 12)	−32.7 ± 0.4 (n = 12)	2.4 ± 0.1 (n = 10)
$NMDG^+$	−27.2 ± 2.1 (n = 8)	0.4 ± 0.2 (n = 8)	−24.4 ± 2.2 (n = 12)	1.3 ± 0.1 (n = 14)	−34.6 ± 0.2 (n = 9)	2.0 ± 0.1 (n = 8)
Anions						
Cl^-	−20.2 ± 2.0 (n = 8)	10.2 ± 0.5 (n = 8)	−21.3 ± 3.1 (n = 20)	6.8 ± 0.2 (n = 15)	−32.4 ± 0.1 (n = 15)	11 ± 0.2 (n = 10)
$Glutamate^-$	−14.4 ± 2.1 (n = 8)	7.0 ± 0.4 (n = 10)	−14.6 ± 3.3 (n = 15)	3.0 ± 0.2 (n = 20)	−27.6 ± 0.8 (n = 9)	7.4 ± 0.2 (n = 11)
$Gluconate^-$	−9.9 ± 1.1 (n = 8)	4.3 ± 0.3 (n = 10)	−13.6 ± 3.2 (n = 15)	2.5 ± 0.1 (n = 20)	−27.2 ± 0.2 (n = 10)	6.9 ± 0.2 (n = 10)

Reversal potentials were analyzed by CLAMPFIT 6 (Axon Instruments). Ionic currents were recorded and calculated by CLAMPEX 5 (Axon Instruments). Data are presented as mean ± standard error (n for numbers of oocytes). Typical recording tracings of currents versus reversal potential and figures of ionic currents in Table 1 are provided in Supplementary Figure 2

[a] The data were adapted from Lin et al. (2006)

Table 2 Relative permeability of cations to K^+ or anions to Cl^-

	MC4100 mbs[a]	SecYEG$^-$ mbs	Recon. SecYEG$^-$ mbs
Na^+/K^+	0.96	0.96	0.99
$NMDG^+/K^+$	0.76	0.89	0.92
Glutamate$^-$/Cl^-	0.77	0.74	0.81
Gluconate$^-$/Cl^-	0.63	0.71	0.79

The relative permeability ratio P_A/P_B was calculated according to the Nernst equation: $E_{rev} = \frac{RT}{zF} \ln \frac{P_A[A]_0}{P_B[B]_0}$, where the gas constant $R = 8.3145$ J mol^{-1} K^{-1}, T is absolute temperature = 297 K (24 °C), z is the charge of the ion, F (Faraday constant) = 96,500 C mol^{-1}. Ion concentrations: K^+ (90 mM), Na^+ (90 mM), $NMDG^+$ (90 mM), Cl^- (90 mM), glutamate$^-$ (80 mM), gluconate$^-$ (80 mM). When the extracellular solution was replaced by glutamate$^-$ or gluconate$^-$, the 10 mM Cl^- remained in the bath solution

[a] The data were adapted from Lin et al. (2006)

using electrophysiological studies with *Xenopus* oocytes. In our previous report, as little as 5 ng of SecA could stimulate ionic currents in *E. coli* wild-type membranes with or without coinjected proOmpA (Lin et al. 2006). However, in membranes depleted of SecYEG from cells or by cholate precipitation, higher amounts of SecA (60–120 ng of current saturation) were needed to stimulate the ionic currents in the oocytes (Fig. 2). LamB signal peptides or precursor proteins can induce the opening of SecA-dependent channels in SecYEG$^-$ membranes in the presence of puromycin, albeit requiring additional SecA, indicating that SecA is sufficient for ion channel activities, even in the absence of detectable SecYEG. This conclusion is further supported by similar findings that SecA liposomes alone can promote protein translocation and channel activity (Hsieh et al. 2011). These data together indicate that SecY is not essential for protein translocation, as determined in both biochemical and electrophysiological studies. Compared to the simpler SecA-liposomal systems (Hsieh et al. 2011), the channel activity of reconstituted membranes is higher and does not require additional ATP (other than those already present in the oocytes). This observation suggests that some other Sec components (e.g., SecDF) or other factors in the membrane can contribute to maintaining the higher channel activity than SecA liposomes alone (Fig. 6). Interestingly, we noted that more SecA was required to recover the ionic currents without SecYEG in either reconstituted membranes or SecA liposomes. It has been reported that SecY contains a high-affinity binding site for SecA (Kim et al. 1994) and a "plug domain" to gate the channel (Li et al. 2007; Tam et al. 2005; Dalal and Duong 2009). The sharp increase in channel activity following the addition of increasing amounts of SecA (Figs. 1c, 2b) suggests a critical concentration for SecA to function in the absence of SecYEG, presumably to form the "low-affinity" SecA channel at higher concentrations (Wang et al. 2003; Hsieh et al. 2011). Such higher SecA concentrations are still within the physiological ranges observed in the cells (as discussed in Hsieh et al. 2011).

Our results show that the SecYEG complex is not essential for the opening of the SecA-dependent channel but is required for efficiency and signal peptide specificity. Proofreading of signal sequences is one of the functions that SecY fulfills in the early stage of protein translocation (Maillard et al. 2007). Wild-type membranes having functional SecYEG rejected the mature precursors' or defective LamB signal peptides' ability to open the protein-conducting channel. Defective LamB signal peptides and OmpA can bypass such recognition and were able to induce the opening of protein-conducting channels without SecYEG. The crystal structure of SecY from *Methanococcus jannaschii* (Bostina et al. 2005; Van den Berg et al. 2004) suggests that SecY possesses a plug domain that is not essential for cell viability in either *E. coli* (Li et al. 2007; Maillard et al. 2007) or yeast (Junne et al. 2006). This SecY plug might be involved in channel gating and specificity of signal peptides (Li et al. 2007; Maillard et al. 2007). Indeed, studies have shown that removal of half or the complete plug domain suppresses the effects of a defective signal sequence of alkaline phosphatase (Li et al. 2007). We have also shown that the channels of these plug mutant membranes lose channel signal peptide specificity (Hsieh et al. 2011). However, in our studies, the proofreading function of the protein-conducting channel cannot be bypassed by a folded OmpA or PhoA or nonspecific unfolded protein (data not shown), indicating that these channels in the absence of SecYEG retain some degree of specificity for secretory proteins. These observations indicate that the gating mechanism is not totally dependent on SecY and may engage other accessory proteins (e.g., SecDFYajC and YidC, which are still present in the membranes we used here). Alternatively, a global structural discrimination by the channel of secretory proteins might be involved. Taken together, our results suggest that SecY does represent a checkpoint for signal sequences or precursor proteins and selects the entrance of the protein-conducting channel proficiently before protein translocation is initiated.

The conductance and ion selectivity of the *E. coli* protein-conducting channels have previously been revealed in several studies (Dalal and Duong 2009; Park and Rapoport 2011; Saparov et al. 2007; Schiebel and Wickner 1992). We have shown that SecA-dependent ion channels in *E. coli* wild-type membrane exhibit selective permeability to monovalent cations and anions. They are more permeable to K^+ and Na^+ than to $NMDG^+$ (Lin et al. 2006). Our

present study shows that the cationic selectivity of the SecA-dependent channels is lost in membranes without SecYEG, suggesting that SecYEG is likely involved in the maintenance of pore sizes or the recognition of ion charges. Interestingly, the anionic permeability is maintained in membranes with or without SecYEG (Tables 1, 2). These data suggest that the SecA protein-conducting channel in the absence of SecYEG may constitute an aqueous environment that is more sensitive to anions than cations during the channel opening, which is similar to SecY plug mutants (Dalal et al. 2010; Dalal and Duong 2009). In addition, the selective ion permeability of the SecA-dependent protein-conducting channels suggests that the interaction of the secretory proteins and the conducting pore could be disrupted in the absence of SecYEG and that SecYEG may play a role in such interaction or in the maintenance of the pore conformations necessary for ion selectivity.

In this report, we provide additional evidence that membranes in the absence of SecYEG are active for the SecA-dependent ion channel but lose the efficiency and specificity of the proofreading function of signal peptides, similar to the SecA-liposome system. However, compared to the SecA liposomes which require additional ATP to function, the channel activity in reconstituted membranes can be fully restored by addition of the SecYEG complex, suggesting that other membrane proteins may contribute to the efficiency of the channel. SecD-SecF-YajC or other membrane proteins may be involved in this apparatus, supporting the basic SecA channel structure for active protein translocation. The use of sensitive assays of electrophysiological voltage-clamping techniques on reconstituted proteoliposomes provides another perspective for studying this dynamic system.

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